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(54) Title: METHODS OF DETECTING TRANSMISSIBLE SPONGIFORM ENCEPHALOPATHIES BY DETECTING 14-3-3 PROTEIN ISOFORMS

(57) Abstract

A method is described for diagnosing transmissible spongiform encephalopathies (TSEs) by detecting the presence and amount of 14-3-3 isoforms in the cerebrospinal fluid of a subject.

*(Referred to in PCT Gazette No. 38/1998, Section II)

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METHODS OF DETECTING TRANSMISSIBLE SPONGIFORM ENCEPHALOPATHIES BY DETECTING 14-3-3 PROTEIN ISOFORMS

STATEMENT OF RIGHTS TO INVENTIONS MADE UNDER FEDERALLY SPONSORED RESEARCH

This invention was made in part during work supported by a grant from the National Institutes of Health NS30531. The government has certain rights in this invention.

FIELD OF THE INVENTION

This invention relates to methods of detecting the presence of transmissible spongiform encephalopathies (TSEs). More specifically it relates to detecting TSEs by determining the presence or amount of 14-3-3 isoforms in the cerebrospinal fluid of a subject.

BACKGROUND OF THE INVENTION

The transmissible spongiform encephalopathies (TSEs) are a group of neurodegenerative diseases. In humans, these diseases include Creutzfeldt-Jakob disease (CJD), Gerstmann-Staussler-Scheinker syndrome, Fatal Familial Insomnia, and Kuru. (*see, e.g.,* Brown *et al.* in NEURODEGENERATIVE DISEASES, Calne *ed.,* W.B. Saunders, Philadelphia (1994); Medori *et al.* (1992) *N. Engl. J. Med.* 326:444-449). In animals, the TSEs include sheep scrapie, bovine spongiform encephalopathy, transmissible mink encephalopathy, and chronic wasting disease of captive mule deer and elk (Gajdusek (1990) *Subacute Spongiform Encephalopathies: Transmissible Cerebral Amyloidoses Caused by Unconventional Viruses.* pp. 2289-2324 In: *Virology,* Fields, ed. New York: Raven Press, Ltd.). All TSEs are characterized by the same hallmarks: a spongiform degeneration, reactive gliosis in the cortical and subcortical gray matters of the brain, and transmission when experimentally inoculated into laboratory animals including primates, rodents and transgenic mice.

The recent international concern about a proposed link between BSE and CJD underscores the need to develop simple, noninvasive, premortem diagnostic tests for both BSE and CJD. Accurate diagnosis of TSEs can currently be performed by brain biopsy or upon postmortem examination. Neither of these option is favorable. Postmortem diagnosis cannot reduce the risk of transmission from living patients and does not address the concerns regarding patient management. Premortem brain biopsy is highly invasive and causes health risks to animals, patients, and health care personnel. Moreover, brain biopsy may often miss the site of pathology.

There remains an urgent need for premortem diagnostic test that can identify transmissible spongiform encephalopathy. Most cerebrospinal fluid (CSF) proteins that have been implicated as pre-mortem diagnostic markers of TSEs have not been very useful diagnositically. For instance, neuron specific enolase (NSE) (Jimi *et al.* (1992) *Lancet*, 211:37-46), S-100b (Jimi *et al., supra*), brain-type isozyme of creatine kinase (Jimi *et al., supra*), GTP binding protein G (Jimi *et al., supra*), ubiquitin (Manaka *et al.* (1992) *Neurosci. Letts.* 139:47-49) and lactic acid (Awerbach *et al.* (1985) *Internat. J. Neurosci.*, 42:1-5.

In animals, the need for a premortem diagnosis is equally acute. Currently, animals diagnosed with BSE are slaughtered and approximately 20% are incorrectly diagnosed. Moreover, a reliable premortem test for clinically affected animals should reduce the risk of possible infection to veterinary pathologists and abbatoir workers by eliminating the need for brain biopsy.

Harrington *et al.* (1986) *N. Engl. J. Med.* 315:279-283 and U.S. Patent No. 4,892,814 reported that two marker proteins, designated proteins 130 and 131 by two dimensional gel electrophoresis (2DE) and silver staining show high specificity and sensitivity for the diagnosis of CJD in humans. These spots migrate at pH 5.2, 26 kDa and pH 5.1, 29 kDa on silver stained 2DE gels have a sensitivity of >98% and a specificity of >99% for CJD. Recently, spots 130/131 have been identified as the brain protein 14-3-3.

The 14-3-3 proteins are a highly conserved group of proteins found in a broad range of species, including plants, yeast, Drosophila and mammals. They exist at low levels in many tissues, but are most highly concentrated in the brain. (see, *e.g.*, Aitken *et al.* (1992) *Trends Biochem. Sci.* 17:498:501). There is increasing evidence that 14-3-3 proteins are critical to cell transformation and mitotic signaling. (see, *e.g.* Ford *et al.*

(1994) *Science* 265:533-535; Freed *et al.* (1994) *Science* 265:1713-1716; Irie *et al.* (1994) *Science* 265:1716-1719 and Fu *et al.* (1994) *Science* 266:126-129). There are at least seven mammalian isoforms of 14-3-3 including, for example, β, γ, δ, ε, θ, ζ and η. (Morrison (1994) *Science* 266:56-57).

It is, however, the surprising discovery of this invention that these various different isoforms of the 14-3-3 family may be specifically diagnostic of TSEs. Polyclonal antibodies against 14-3-3 (all isoforms), 14-3-3γ, 14-3-3β, and 14-3-3θ are immunoreactive with a 30 kDa marker band from CJD CSF. However, 14-3-3ε and 14-3-3ζ antibodies are not present in TSE-positive samples. This invention also describes for the first time how antibodies to 14-3-3 isoforms is a pre-mortem diagnostic for BSE in cattle.

SUMMARY OF THE INVENTION

The present invention provides methods of diagnosing transmissible spongiform encephalopathies by detecting the 14-3-3 isoforms in the cerebrospinal fluid of an animal.

As will become apparent, preferred features and characteristics of one aspect of the invention are applicable to any other aspect of the invention.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a half-tone reproduction of a silver-stained 2DE gel of cerebrospinal fluid from a patient with Creutzfeldt-Jakob disease. Spot 130 and 131 are denoted with arrows and correlate specifically with spongiform pathology in patients affect by Creutzfeldt-Jakob disease.

Figure 2, panels A through F, are half-tone reproductions of an immunostained one-dimensional SDS-PAGE blots with polyclonal antibodies to 14-3-3 isoforms. In each panel, lane 1 contains cerebrospinal fluid from a patient with Creutzfeldt-Jakob disease and lane 2 contains the cerebrospinal fluid from a normal individual. Panel A shows staining with anti-14-3-3 (all isoforms); Panel B shows staining with anti-14-3-3γ; Panel C shows anti-14-3-3β; Panel D shows anti-14-3-3θ; Panel E shows anti-14-3-3ε; Panel F shows anti-14-3-3ζ. The diagnostic band migrates at 30 kDa as shown by the arrows.

Figure 3 is a half-tone reproduction of 2DE gel of cerebrospinal fluid from a bovine affected by bovine spongiform encephalopathy. The region marked in the box represents

the region that should contain spots 130/131. No spots comigrating with human cerebrospinal fluid 130/131 are seen.

Figure 4 is a half-tone reproduction of 1D gels on two bovine cerebrospinal fluid samples blotted onto PVDF or nitrocellulose. The 14-3-3γ antibody immunostained 14-3-3 in bovine cerebrospinal fluid blotted to PVDF but not material blotted to nitrocellulose. The diagnostic band migrates at approximately 30 kDa by SDS-PAGE.

Figure 5 is a half-tone reproduction of a 1D gel of bovine samples blotted onto PVDF membrane and immunostained with 14-3-3γ antibody. Lane 1 is a sample from normal cow brain extract, lanes 2-5 are premortem samples from cerebrospinal fluid from BSE-affected cattle and lanes 6-9 are spinal fluid samples from control cattle (non-BSE-affected). The 14-3-3 band migrates at a position corresponding to 30 kDa and its appearance correlates with BSE pathology.

DETAILED DESCRIPTION OF THE INVENTION

Throughout this application, various publications, patents, and published patent applications are referred to by an identifying citation. The disclosure of the publications, patents, and published patent specifications referenced in this application are hereby incorporated by reference into the present disclosure to more fully describe the state of the art to which this invention pertains.

As used herein, certain terms will be used which have defined meanings.

The term "14–3–3 protein" is used herein to refer to mean any member of isoform of the 14–3–3 class of proteins as it is commonly known to those of skill in the art *(see, e.g.,* Ichimura *et al.* (1988) *Proc. Nat'l, Acad. Sci., USA* 85:7084–7088; Zupan *et al.* (1992) *J Biol. Chem.*, 267, 8707–87 10; Aitken *et al.* (1992) *Trends. Biochem. Sci.,* 17: 498–501 Burbtlo *et al.* (1995) *Current Biology,*5:95–96; Robinson *et al.* (1994) *Biochem. J.* 299: 853–861; Ichimura *et al.* (1988) *Proc. Nat'l. Acad. Sci., USA,* 85: 7084–7088; and Morgan *et al.* (1992) *Nature,* 355: 833–836. Assays that detect 14–3–3 are intended to detect the level of endogenous (native) 14–3–3 present in subject biological sample (*e.g.,* CSF). However, exogenous 14–3–3 (14–3–3 protein from a source extrinsic to the biological sample) may be added to various assays to provide a label or to compete with the native 14–3–3 in binding to an anti–14–3–3 antibody. One of skill will appreciate that a 14–3–3 mimetic may be used in place of exogenous 14–3–3 in this context. An "14–3–3 mimetic",

as used herein, refers to a molecule that bears one or more 14–3–3 epitopes such that it is specifically bound by an antibody that specifically binds native 14–3–3.

As used herein, the term "glycosylation" is intended to mean the covalent addition of sugar or sugar-related molecules to any protein or polynucleotide. Thus, a glycosylated protein has one or more covalently bound sugar-related molecules and one protein can have multiple glycosylated forms. Thus, the terms "glycoform" and "isoforms" are used interchangeably and are intended to mean to the different glycosylated variants of one protein. The glycoform may be identified herein by its isoelectric focusing point (pI) and its molecular weight (MW) in kilodaltons (kD). The pI and molecular weight values are intended to include a range which includes any variations which may occur due to experimental conditions as would be known by one of skill in the art. In the neutral pH region, pIs will generally range up or down 0.5 from the value given. At higher pHs, the range may be $\pm$ 1. Molecular weight ranges will usually be within 5 kilodaltons of the value given, although glycoproteins may migrate anomalously in SDS PAGE gels and give show MW values which are higher than the actual MW.

As used herein, the term "cerebrospinal fluid" or "CSF" is intended to include whole cerebrospinal fluid or derivatives or fractions thereof well known to those of skill in the art. Thus, a cerebrospinal fluid sample can include various fractionated forms of cerebrospinal fluid or can include various diluents added to facilitate storage or processing in a particular assay. Such diluents are well known to those of skill in the art and include various buffers, preservatives and the like.

As used herein, the term "2-dimensional gel electrophoresis" or "2-D gel electrophoresis" or "2DE" is intended to refer to the two dimensional migration of proteins in solution of suspension in the presence of an electrical field. Methods of 2DE are well known to those of skill in the art. See generally, R. Scopes (1982) *Protein Purification*, Springer-Verlag, N.Y. and Deutscher (1990) *Methods in Enzymology Vol. 182: Guide to Protein Purification*, Academic Press Inc., N.Y.). For example, 2DE relies on isoelectric focusing (IEF) in either carrier ampholyte gradient gels or immobilized pH gradients for one dimension and SDS-polyacrylamide gels for the second dimension. The amount of polyacrylamide to be used in making the gels can be readily determined by a skilled artisan.

As used herein, the term "synthetic peptide" refers to any amino acid or polypeptide which results from chemical synthesis or assembly. Methods of synthesizing peptides are known in the art and include for example, solution or solid phase peptide synthesis of either the t-Boc or Fmoc types. Methods of synthesizing peptides may be found, for example, in *Current Protocols in Molecular Biology*, Ausubel *et al.* eds. (1996) §11.14.

As used herein, an "immunoassay" is an assay that utilizes an antibody to specifically bind to the analyte. The immunoassay is characterized by the use of specific binding to a particular antibody as opposed to other physical or chemical properties to isolate, target, and quantify the analyte.

As used herein, an "antibody" refers to a protein consisting of one or more polypeptides substantially encoded by immunoglobulin genes or fragments of immunoglobulin genes. The recognized immunoglobulin genes include the kappa, lambda, alpha, gamma, delta, epsilon and mu constant region genes, as well as the myriad immunoglobulin variable region genes. Light chains are classified as either kappa or lambda. Heavy chains are classified as gamma, mu, alpha, delta, or epsilon, which in turn define the immunoglobulin classes, IgG, IgM, IgA, IgD and IgE, respectively.

The basic immunoglobulin (antibody) structural unit is known to comprise a tetramer. Each tetramer is composed of two identical pairs of polypeptide chains, each pair having one "light" (about 25 kD) and one "heavy" chain (about 50–70 kD). The N–terminus of each chain defines a variable region of about 100 to 110 or more amino acids primarily responsible for antigen recognition. The terms variable light chain (V_l) and variable heavy chain (V_H) refer to these variable regions of the light and heavy chains respectively.

Antibodies may exist as intact immunoglobulins or as a number of well characterized fragments produced by digestion with various peptidases. Thus, for example, pepsin digests an antibody below the disulfide linkages in the hinge region to produce F(ab)'$_2$, a dimer of Fab which itself is a light chain joined to V_H–C_H1 by a disulfide bond. The F(ab)'$_2$ may be reduced under mild conditions to break the disulfide linkage in the hinge region thereby converting the F(ab)'$_2$ dimer into an Fab' monomer. The Fab' monomer is essentially an Fab with part of the hinge region (*See*, *Fundamental Immunology*, W.E. Paul, ed., Raven Press, N.Y. (1993) for a more detailed description of

other antibody fragments). While various antibody fragments are defined in terms of the digestion of an intact antibody, one of skill will appreciate that such Fab' fragments may be synthesized *de novo* either chemically or by utilizing recombinant DNA methodology. Thus, the term antibody, as used herein also includes antibody fragments either produced by the modification of whole antibodies or synthesized *de novo* using recombinant DNA methodologies.

The phrase "specifically binds to a protein" or "specifically immunoreactive with", when referring to an antibody refers to a binding reaction which is determinative of the presence of the protein in the presence of a heterogeneous population of proteins and other biologics. Thus, under designated immunoassay conditions, the specified antibodies bind to a particular protein and do not bind in a significant amount to other proteins present in the sample. Specific binding to a protein under such conditions may require an antibody that is selected for its specificity for a particular protein. For example, antibodies can be raised to the human 14-3-3 protein that bind 14-3-3 and not to any other proteins present in a biological sample (*e.g.*, CSF). A variety of immunoassay formats may be used to select antibodies specifically immunoreactive with a particular protein. For example, solid–phase ELISA immunoassays are routinely used to select monoclonal antibodies specifically immunoreactive with a protein. *See* Harlow and Lane (1988) *Antibodies. A Laboratory Manual,* Cold Spring Harbor Publications, New York, for a description of immunoassay formats and conditions that can be used to determine specific immunoreactivity.

As used herein, the term "subject" refers a mammal and includes, but is not limited to, humans, bovine, mink, sheep, elk and deer.

Detection of Transmissible Spongiform Encephalopathies

This invention provides methods for the detection of transmissible spongiform encephalopathies (TSEs) by detecting the presence or absence or quantification of a 14-3-3 protein in a sample of cerebrospinal fluid (CSF). While the 14-3-3 proteins were previously known, it is the surprising discovery of this invention that different 14-3-3 isoforms can be diagnostic of TSE. The detection methods of this invention thus provide new uses for this previously known class of proteins.

Using the methods of the present invention, TSEs can be diagnosed by premortem testing. Table 1 shows the sensitivity and specificity of immunoassays for 14-3-3γ isoform antibodies in immunostained one-dimensional gels (1D). Table 2 shows the sensitivity and specificity of immunoassays for 14-3-3γ isoform antibodies in immunostained two-dimensional gels (2DE).

Table 1

Diagnosis	Total Positive	Total Sample
Sporadic CJD	68	71
New Variant CJD	2	2
Other Dementias	1	91
Experimentally induced TSEs in animals	26	30
Control animals	1	94

Table 2

Diagnosis	Total Positive	Total Samples
Sporadic CJD	69	70
New Variant CJD	4	4
Other Dementias	1	298

Tables 1 and 2 show that 14-3-3γ antibodies are both a sensitive and specific method of detecting TSEs.

In another aspect, the invention provides methods for TSEs by determining whether specific 14-3-3 isoforms are present or absent in CSF. For example, antibodies to 14-3-3 ε and ζ, do not detect the 30 kDa band diagnostic of TSEs. In contrast, 14-3-3γ, β and θ are diagnostic of TSEs. As detailed below in the Experimental section, these different isoforms can therefore be used to diagnose TSEs.

Detection of Animal TSEs with 14-3-3 Isoforms

This invention also provides for methods of diagnosing animal TSEs by detecting 14-3-3 isoforms. As shown in Figure 5, and detailed below, the CSF from all ten animals later confirmed positive for BSE, had a 14-3-3 immunoreactive band. The 14-3-3 isoform

antibody was not reactive with CSF samples obtained from non-BSE or with an extract of normal bovine brain.

Assays for 14-3-3 Proteins

As indicated above, it was a discovery of this invention that nervous system diseases can be detected by the presence and amount of 14-3-3 isoforms in cerebrospinal fluid from animals and humans. The 14-3-3 proteins can be detected and quantified by any of a number of means well known to those of skill in the art. These may include analytic biochemical methods such as electrophoresis, capillary electrophoresis, high performance liquid chromatography (HPLC), thin layer chromatography (TLC), hyperdiffusion chromatography, mass spectrometry and the like, or various immunological methods such immunodiffusion (single or double), immunoelectrophoresis, radioimmunoassay(RIA), enzyme–linked immunosorbent assays (ELISAs), immunofluorescent assays, western blotting, and the like.

In a preferred embodiment, the 14-3-3 proteins are detected in an electrophoretic protein separation, more preferably in a two–dimensional electrophoresis, while in a most preferred embodiment, the 14-3-3 proteins are detected using an immunoassay. The collection of biological samples and subsequent testing for 14-3-3 proteins is discussed in more detail below.

A) Sample Collection and Processing

The 14-3-3 proteins are preferably quantified in cerebrospinal fluid derived from a mammal, including human patients and animals. Obtaining and storing CSF are well known to those of skill in the art. Typically CSF is obtained by lumbar puncture. The CSF may be diluted by the addition of buffers or other reagents well known to those of skill in the art and may be stored for up to 24 hours at 2–8°C, or at –20°C or lower for longer periods, prior to measurement of 14-3-3. Any of a number of standard aqueous buffer solutions, employing one of a variety of buffers, such as phosphate, Tris, or the like, at physiological pH can be used. In a preferred embodiment, the CSF is stored at –70°C without preservative indefinitely.

B) Electrophoretic Assays

As indicated above, the presence or absence of 14-3-3 isoforms in cerebrospinal fluid can be determined using electrophoretic methods. Means of detecting proteins using electrophoretic techniques are well known to those of skill in the art (see generally, R. Scopes (1982) *Protein Purification,* Springer–Verlag, N.Y.; Deutscher, (1990) *Methods in Enzymology Vol. 182: Guide to Protein Purification.,* Academic Press, Inc., N.Y.). In one embodiment, the 14-3-3 proteins are detected using two-dimensional electrophoresis. A particularly preferred separation relies on isoelectric focusing (IEF) in carrier ampholyte gradient gels or immobilized pH gradients for one dimension and 14% polyacrylamide gels for the second dimension. A detailed protocol for 14-3-3 isolation using two-dimensional electrophoresis is provided in Examples below.

Proteins separated on 2DE gels can be visualized by any method known in the art. For example, Coomassie blue staining, gold staining, silver staining and the like may be used to visualize proteins directly on the gels. (see, generally, *Current Protocols in Molecular Biology*, Ausubel *et al.* eds. (1996) § 10.6 to 10.8 and references therein). Alternatively, the proteins on the 2DE gel can be transferred or blotted onto a membrane and probed with an antibody. Methods of blotting and immunostaining are known to those of skill in the art. (see, e.g., Ausubel (1996), *supra*, § 10.8). In a preferred embodiment, the present invention detects the presence and amount of 14-3-3 first by silver staining and, subsequently, by immunostaining.

C) Immunological Binding Assays

In another embodiment, the 14-3-3 proteins are detected or quantified in CSF using any of a number of well recognized immunological binding assays *(see, e.g.,* U.S. Patents 4,366,241; 4,376,110; 4,517,288; and 4,837,168). For a review of the general immunoassays, see also *Methods in Cell Biology Volume 37. Antibodies in Cell Biology,* Asai, ed. Academic Press, Inc. New York (1993); *Basic and Clinical Immunology* 7th Edition, Stites & Terr, eds. (1991).

Immunological binding assays (or immunoassays) typically utilize a "capture agent" to specifically bind to and often immobilize the analyte (in this case 14-3-3). The

capture agent is a moiety that specifically binds to the analyte. In a preferred embodiment, the capture agent is an antibody that specifically binds 14-3-3 protein(s).

The antibody (anti–14-3-3) may be produced by any of a number of means well known to those of skill in the art (see, *e.g. Methods in Cell Biology Volume 37 Antibodies in Cell Biology*, Asai, ed. Academic Press, Inc. New York (1993); and *Basic and Clinical Immunology* 7th Edition, Stites & Terr, eds. (1991)). The antibody may be a whole antibody or an antibody fragment. It may be polyclonal or monoclonal, and it may be produced by challenging an organism (*e.g.* mouse, rat, rabbit. *etc.)* with a 14-3-3 protein or an epitope derived therefrom. Alternatively, the antibody may be produced de *novo* using recombinant DNA methodology. The antibody can also be selected from a phage display library screened against 14-3-3 (see, *e.g.* Vaughan *et al.* (1996) *Nature Biotechnology,* 14:309–314 and references therein). Commercially available 14-3-3 isoform antibodies are also available from, for example, Santa Cruz Biotechnology.

Immunoassays also often utilize a labeling agent to specifically bind to and label the binding complex formed by the capture agent and the analyte, The labeling agent may itself be one of the moieties comprising the antibody/analyte complex. Thus, the labeling agent may be a labeled 14-3-3 protein or a labeled anti–14-3-3 antibody, Alternatively, the labeling agent may be a third moiety, such as another antibody, that specifically binds to the antibody/14-3-3 complex.

In a preferred embodiment, the labeling agent is a second human 14-3-3 antibody bearing a label. Alternatively, the second 14-3-3 antibody may lack a label, but it may, in turn, be bound by a labeled third antibody specific to antibodies of the species from which the second antibody is derived. The second can be modified with a detectable moiety, such as biotin, to which a third labeled molecule can specifically bind, such as enzyme–labeled streptavidin.

Other proteins capable of specifically binding immunoglobulin constant regions, such as protein A or protein G may also be used as the label agent. These proteins are normal constituents of the cell walls of streptococcal bacteria. They exhibit a strong non–immunogenic reactivity with immunoglobulin constant regions from a variety of species. *See*, generally Kronval, *et al. J. Immunol.;* 111:1401–1406 (1973), and Akerstrom, *et al., J. Immunol.,* 135:2589–2542 (1985).

Throughout the assays, incubation and/or washing steps may be required after each combination of reagents. Incubation steps can vary from about 5 seconds to several hours, preferably from about 5 minutes to about 24 hours. However, the incubation time will depend upon the assay format, analyte, volume of solution, concentrations, and the like. Usually, the assays will be carried out at ambient temperature, although they can be conducted over a range of temperatures, such as 10°C to 40°C.

1. Non–Competitive Assay Formats

Immunoassays for detecting CSF 14-3-3 protein may be either competitive or noncompetitive. Noncompetitive immunoassays are assays in which the amount of captured analyte (in this case 14-3-3) is directly measured. In one preferred "sandwich" assay, for example, the capture agent (anti–14-3-3 antibodies) can be bound directly to a solid substrate where they are immobilized. These immobilized antibodies then capture 14-3-3 present in the test sample. The 14-3-3 thus immobilized is then bound by a labeling agent, such as a second human 14-3-3 antibody bearing a label. Alternatively, the second 14-3-3 antibody may lack a label, but it may, in turn, be bound by a labeled third antibody specific to antibodies of the species from which the second antibody is derived. The second can be modified with a detectable moiety, such as biotin, to which a third labeled, molecule can specifically bind, such as enzyme–labeled strepavidin.

2. Competitive Assay Formats

In competitive assays, the amount of analyte (14-3-3) present in the sample is measured indirectly by measuring the amount of an added (exogenous) analyte (14-3-3) displaced (or competed away) from a capture agent (anti 14-3-3 antibody) by the analyte present in the sample. In one competitive assay, a known amount of, in this case, 14-3-3 is added to the sample and the sample is then contacted with a capture agent, in this case an antibody that specifically binds 14-3-3. The amount of 14-3-3 bound to the antibody is inversely proportional to the concentration of 14-3-3 present in the sample before the exogenous 14-3-3 is added to the reaction mix.

In a particularly preferred embodiment, the antibody is immobilized on a solid substrate. The amount of 14-3-3 bound to the antibody may be determined either by measuring the amount of 14-3-3 present in an 14-3-3/antibody complex, or alternatively by

measuring the amount of remaining uncompeted 14-3-3. The amount of 14-3-3 may be detected by providing a labeled 14-3-3 molecule.

A hapten inhibition assay is another preferred competitive assay. In this assay a known analyte, in this case 14-3-3 is immobilized on a solid substrate. A known amount of anti–14-3-3 antibody is added to the sample, and the sample is then contacted with the immobilized 14-3-3. In this case, the amount of anti–14-3-3 antibody bound to the immobilized 14-3-3 is inversely proportional to the amount of 14-3-3 present in the sample. Again the amount of immobilized antibody may be detected by detecting either the immobilized fraction of antibody or the fraction of the antibody that remains in solution. Detection may be direct where the antibody is labeled or indirect by the subsequent addition of a labeled moiety that specifically binds to the antibody as described above.

3. Other Assay Formats

In a preferred embodiment, Western blot (immunoblot) analysis is used to detect and quantify the presence of 14-3-3 in the sample. The technique generally comprises separating sample proteins by gel electrophoresis on the basis of molecular weight, transferring the separated proteins to a suitable solid support, (such as a nitrocellulose filter, a nylon filter, or derivatized nylon filter), and incubating the sample with the antibodies that specifically bind 14-3-3. The anti–14-3-3 antibodies specifically bind to 14-3-3 on the solid support. These antibodies may be directly labeled or alternatively may be subsequently detected using labeled antibodies (*e.g.*, labeled sheep anti–mouse antibodies) that specifically bind to the anti-14-3-3. A particularly preferred protocol for Western Blot detection of human or animal 14-3-3 protein in CSF is provided in Example 1.

Other assay formats include liposome immunoassays (LIA), which use liposomes designed to bind specific molecules (*e.g.*, antibodies) and release encapsulated reagents or markers. The released chemicals are then detected according to standard techniques *(see, Monroe et at.* (1986) *Amer. Clin. Prod. Rev.* 5:34–41).

E) Labels

The particular label or detectable group used in the assay is not a critical aspect of the invention, so long as it does not significantly interfere with the specific binding of the antibody used in the assay. The detectable group can be any material having a detectable physical or chemical property. Such detectable labels have been well developed in the field of immunoassays and, in general, most any label useful in such methods can be applied to the present invention. Thus, a label is any composition detectable by spectroscopic, photochemical, biochemical, immunochemical, electrical, optical or chemical means. Useful labels in the present invention include magnetic beads (*e.g.* Dynabeads™), fluorescent dyes (*e.g.*, fluorescein isothiocyanate, texas red, rhodamine, and the like), radiolabels (*e.g.*, ^{3}H, ^{125}I, ^{35}S, ^{14}C, or ^{32}P), enzymes (*e.g.*, horse radish peroxidase, alkaline phosphatase and others commonly used in an ELISA), and colorimetric labels such as colloidal gold or colored glass or plastic (*e.g.* polystyrene, polypropylene, latex, *etc.)* beads.

The label may be coupled directly or indirectly to the desired component of the assay according to methods well known in the art. As indicated above, a wide variety of labels may be used, with the choice of label depending on sensitivity required, ease of conjugation with the compound, stability requirements, available instrumentation, and disposal provisions.

Non–radioactive labels are often attached by indirect means. Generally, a ligand molecule (*e.g.*, biotin) is covalently bound to the molecule. The ligand then binds to an anti–ligand (*e.g.*, streptavidin) molecule which is either inherently detectable or covalently bound to a signal system, such as a detectable enzyme, a fluorescent compound, or a chemiluminescent compound. A number of ligands and anti–ligands can be used. Where a ligand has a natural anti–ligand, for example, biotin, thyroxine, and cortisol, it can be used in conjunction with the labeled, naturally occurring anti–ligands. Alternatively, any haptenic or antigenic compound can be used in combination with an antibody.

The molecules can also be conjugated directly to signal generating compounds, *e.g.*, by conjugation with an enzyme or fluorophore. Enzymes of interest as labels will primarily be hydrolases, particularly phosphatases, esterases and glycosidases, or oxidoreductases, particularly peroxidases. Fluorescent compounds include fluorescein and its derivatives, rhodamine and its derivatives, dansyl, umbelliferone, etc.

Chemiluminescent compounds include luciferin, and 2,3–dihydrophtlialazinediones, *e.g.*, luminol. (For a review of various labeling or signal producing systems which may be used, *see*, U.S. Patent No. 4,391,904).

Means of detecting labels are well known to those of skill in the art. Thus, for example, where the label is a radioactive label, means for detection include a scintillation counter or photographic film as in autoradiography. Where the label is a fluorescent label, it may be detected by exciting the fluorophore with the appropriate wavelength of light and detecting the resulting fluorescence. The fluorescence may be detected visually, by means of photographic film, by the use of electronic detectors such as charge coupled devices (CCDs) or photomultipliers and the like. Similarly, enzymatic labels may be detected by providing the appropriate substrates for the enzyme and detecting the resulting reaction product. Finally simple colorimetric labels may be detected simply by observing the color associated with the label. Thus, in various dipstick assays, conjugated gold often appears pink, while various conjugated beads appear the color of the bead.

Some assay formats do not require the use of labeled components. For instance, agglutination assays can be used to detect the presence of the target antibodies. In this case, antigen–coated particles are agglutinated by samples comprising the target antibodies. In this format, none of the components need be labeled and the presence of the target antibody is detected by simple visual inspection.

F) Substrates

As mentioned above, depending upon the assay, various components, including the antigen, target antibody, or anti–human antibody, may be bound to a solid surface. Many methods for immobilizing biomolecules to a variety of solid surfaces are known in the art. For instance, the solid surface may be a membrane (*e.g.*, nitrocellulose), a microtiter dish (*e.g.*, PVC, polypropylene, or polystyrene), a test tube (glass or plastic), a dipstick (*e.g.* glass, PVC, polypropylene, polystyrene, latex, and the like), a microcentrifuge tube, or a glass or plastic bead. The desired component may be covalently bound or noncovalently attached through nonspecific bonding.

A wide variety of organic and inorganic polymers, both natural and synthetic may be employed as the material for the solid surface. Illustrative polymers include polyethylene, polypropylene, poly(4-methylbutene), polystyrene, polymethacrylate,

poly(ethylene terephthalate), rayon, nylon, poly(vinyl butyrate), polyvinylidene difluoride (PVDF), silicones, polyformaldehyde, cellulose, cellulose acetate, nitrocellulose, and the like. Other materials which may be employed, include paper, glasses, ceramics, metals, metalloids, semiconductive materials, cements or the like. In addition, are included substances that form gels, such as proteins (*e.g.*, gelatins), lipopolysaccharides, silicates, agarose and polyacrylamides can be used. Polymers which form several aqueous phases, such as dextrans, polyalkylene glycols or surfactants, such as phospholipids, long chain (12–24 carbon atoms) alkyl ammonium salts and the like are also suitable. Where the solid surface is porous, various pore sizes may be employed depending upon the nature of the system.

In preparing the surface, a plurality of different materials may be employed, particularly as laminates, to obtain various properties. For example, protein coatings, such as gelatin can be used to avoid non–specific binding, simplify covalent conjugation, enhance signal detection or the like.

If covalent bonding between a compound and the surface is desired, the surface will usually be polyfunctional or be capable of being polyfunctionalized. Functional groups which may be present on the surface and used for linking can include carboxylic acids, aldehydes, amino groups, cyano groups, ethylenic groups, hydroxyl groups, mercapto groups and the like. The manner of linking a wide variety of compounds to various surfaces is well known and is amply illustrated in the literature. *See*, for *example, Immobilized Enzymes,* Ichiro Chibata, Halsted Press, New York, 1978, and Cuatrecasas (1970) *J. Biol. Chem.* 245: 3059).

In addition to covalent bonding, various methods for noncovalently binding an assay component can be used. Noncovalent binding is typically nonspecific absorption of a compound to the surface. Typically, the surface is blocked with a second compound to prevent nonspecific binding of labeled assay components. Alternatively, the surface is designed such that it nonspecifically binds one component but does not significantly bind another. For example, a surface bearing a lectin such as Concanavalin A will bind a carbohydrate containing compound but not a labeled protein that lacks glycosylation. Various solid surfaces for use in noncovalent attachment of assay components are reviewed in U.S. Patent Nos. 4,447,576 and 4,254,082.

The following examples are provided to illustrate but not limit the present invention.

EXPERIMENTAL

Example 1: Analysis of 14-3-3 Isoforms in Cerebrospinal Fluid (CSF)

Sample preparation

Cerebrospinal fluid samples were kindly provided by various neurologists and veterinarians. The diagnoses were made by the referring physicians according to standard clinical criteria in addition to pathological studies as appropriate. The CSF was collected and the samples were immediately frozen at -70°C before shipment to our laboratory. Upon receipt, samples were thawed and aliquoted for use. Samples for 2-DE were prepared by the addition of 9M urea, 2% 2-mercaptoethanol, 2% NP-40, 0.8% BioLyte pH 3-10 carrier ampholytes, and 0.002% Bromophenol blue. For SDS-PAGE, 50 µL CSF was added to 20 µL Laemmli sample buffer, heated for 5 minutes at 95°C, and loaded.

Two-dimensional electrophoresis of proteins.

Samples were separated by two-dimensional electrophoresis and silver stained as previously described Harrington *et al.* (1991) *Methods: A Companion to Methods in Enzymology* 3:135-141. Briefly, broad-range IEF was performed with carrier ampholytes in 16 cm long, 1.4 mm diameter tube gels for 13,000 volt-hours. SDS-PAGE was achieved in 1.5 mm thick, 16 cm x 20 cm vertical slab gels.

The 14-3-3 SDS-PAGE immunoassay for CJD.

The 14-3-3 SDS-PAGE immunoassay was performed according to a method adapted from Brown *et al.* (1986) *N. Engl. J. Med.* 314:547-551. Fifty microliters of cerebrospinal fluid was mixed with 10 microliters of sample buffer (5% glycerol, 1% 2-mercaptoethanol, 1% sodium dodecyl sulfate, and a trace of bromophenol blue in the final solution) and boiled for 5 minutes. Samples were separated by sodium dodecyl sulfate-polyacrylamide-gel electrophoresis (4% stacking gel with 12 percent resolving gel at 75 V for three hours). Proteins were blotted to either PVDF (Millipore) or nitrocellulose (Schleicher and Schuell). The polyclonal 14-3-3 antibodies used were purchased from

Santa Cruz Biotechnology (catalog numbers: sc-629, sc-628, sc-731, sc-732, sc-1020, and sc-1019) and were diluted 1:500. The anti-rabbit-horse radish peroxidase-conjugated antibody (Promega) was diluted 1:5000. With the exception of final washes, all steps during the immunodetection were performed in the presence of 5% milk in TBST. Chemiluminescent detection (Pierce) on Kodak XAR film was used.

Figure 1 shows results of the silver stained 2-DE gel pattern of CSF taken from a patient diagnosed with CJD. Spots 130 and 131 are denoted by the arrows and these spots selectively appear in the CSF of patients with CJD. In an attempt to develop an immunoassay for CJD, these spots were characterized. Amino acid sequencing of comigrating spots from normal human brain has revealed that these spots are proteins which belong to the 14-3-3 family of proteins. The amino acid sequence data suggests that 130/131 are derived from 14-3-3γ. However, we describe herein for the first time antibodies tested against various isoforms of the 14-3-3 family of proteins for use with an SDS-PAGE immunoassay because of the sequence similarity among these isoforms.

14-3-3 isoform antibodies recognize a 30 kDa band on 1D protein gels

Figure 2 depicts the results obtained with antibodies specific to different 14-3-3 isoforms in CJD screening. In all panels, lane 1 is CSF obtained from a patient diagnosed with CJD and lane 2 is CSF from a control patient. The CJD marker band of interest migrates at 30 kDa by SDS-PAGE. Panel A depicts the results with an antibody used to detect all isoforms of 14-3-3 and it is mildly reactive at 30 kDa. Panels B, C, and D utilize antibodies against the γ, β, and θ isoforms of 14-3-3, respectively. All of these antibodies are reactive with the 30 kDa band. Panels E and F show the use of antibodies against 14-3-3 ε and ζ, respectively and, interestingly, both of these antibodies do not detect any 30 kDa band. Taken together, these results suggest that specific isoforms may be found in CJD CSF. Previous studies on 14-3-3 isoforms in rat brain (see Martin *et al.* (1994) *J. Neurochem.* 63:2259-2265) show that a distribution of isoforms among different membrane preparations and subcellular fractions exists. The onset of spongiform pathology may result in the specific release of certain isoforms which is consistent with the observed results from Figure 2.

Furthermore, it has been shown that the 14-3-3 family of proteins forms both homodimers and heterodimers among its isoforms. (Aitken *et al.* (1995) *Biochem. Soc.*

Trans. 23:605-611). The positive results obtained with the and γ, β, and θ antibodies may reflect the release of specific multimers of the 14-3-3 family from neuronal tissue. Among the isoforms tested, the γ isoform currently demonstrates the highest correlation to a diagnosis of CJD and clinical pathology.

Example 2: Analysis of Cattle Cerebrospinal fluid with 14-3-3 Isoform Antibodies

2-DE was performed on cattle CSF in an attempt to identify a 130/131-like marker in CSF obtained from bovines affected by BSE. Figure 3 depicts a typical silver stained 2D gel from BSE CSF. A region is marked in Figure 3 to denote the region in which 130/131-like spots would most probably appear. No such spots were detected in this region of BSE CSF 2D gels. Based on our hypothesis that spongiform degeneration results in leakage of 130/131 into human CSF, we would expect comigrating spots to appear in BSE CSF. Our inability to detect such spots is likely a result of either the low abundance of 14-3-3 in BSE CSF or their altered position in bovine as compared to human reference CSF proteins. Further refinement of our sample preparation and separation technique would enable us to detect these spots.

The specificity of human 14-3-3 isoform antibodies was also tested on bovine samples. The human and bovine sequences for several of the 14-3-3 isoforms are similar. In particular, the 14-3-3γ isoform is nearly identical for human and bovines. The human sequence has an additional asparagine residue at the C-terminus (A. Aitken, personal communication). The polygonal antibody obtained (Santa Cruz Biotechnology) is antigenic against the C-terminal sequence of human 14-3-3γ. Figure 4 depicts our results with a 14-3-3γ immunostain against 2 samples of bovine CSF. When these proteins are blotted to PVDF (Millipore), the antibody is able to recognize the 30 kDa band of interest. However, when these same proteins are blotted to nitrocellulose (Schleicher and Schuell) the antibody is unable to recognize any 14-3-3. These results were consistent for all bovine samples studied. Thus, PVDF is the material of choice for use in an immunostain with this antibody. The observed differences between these materials may be the result of the conformation which the bovine 14-3-3 takes when interacting with either PVDF or nitrocellulose. Binding to PVDF allows a conformation which the anti-human-14-3-3γ antibody.

The present invention describes how the presence of 14-3-3 in CJD CSF can be detected with antibodies against several isoforms. 14-3-3 can be detected in bovine CSF using the same 14-3-3γ antibody when these proteins are blotted to PVDF. This has led to the development of a method of diagnosing TSEs which is even more simple than 2-DE. Among bovines, this 14-3-3 isoform immunoassays can pave the way for even simpler methods for diagnosing BSE-affected cattle and scrapie-infected sheep.

Example 3: 14-3-3 Isoforms and Bovine Spongiform Encephalitis (BSE)

We describe herein for the first time premortem 14-3-3 levels from clinically affected bovines with BSE. Normal bovine brain was obtained from Sierra Medical Science (California). All CSF samples were obtained after barbiturate anesthesia and were kindly provided by the Ministry of Agriculture, Fisheries and Food (United Kingdom). Spinal fluid from ten samples with clinical features of BSE were obtained. All ten animals were verified at autopsy to have BSE. Controls were spinal fluid samples collected from six British animals which were fed neither meat nor bone meal and were BSE-negative at autopsy.

Samples were analyzed essentially as described in Example 1. CSF samples were stored at -70°C to minimize protein degradation. 50 microliters of CSF was mixed with 20 microliters Laemmli SDS buffer and heated for 5 minutes. A crude extract of bovine brain was prepared by methods known in the art. 10 micrograms of protein in Laemmli buffer was loaded into each lane of a 12%T polyacrylamide gel, transferred to PVDF membrane and immunostained. The 14-3-3γ antibody (Santa Cruz Biotechnology), which is specific for the γ isoform of 14-3-3 was used for detection. Moreover, as described in Example 2, the electrophoresed proteins were blotted onto PVDF (Millipore) material. Enhanced chemiluminescence (Pierce) was used for detection.

As shown in Figure 5, a 30 kDa, 14-3-3 immunoreactive band from an extract of normal bovine brain (lane 1) has the same mobility as a band from BSE samples (lanes 2 through 5) that is not seen in control CSF (lanes 6 through 9). All ten animals with BSE had this band while it was absent in five of six controls. The one control sample which had a small amount of 14-3-3 was believed to be the result of technical error.

These results represent the first experimental evidence that a 14-3-3 immunoassay can aid in the premortem diagnosis of BSE in clinically-affected cattle. The 14-3-3 immunoassay shows good sensitivity (10/10).

It should be understood that the examples and embodiments described herein are for illustrative purposes only and that various modifications or changes in light thereof will be suggested to persons skilled in the art and are to be included within the spirit and purview of this application and scope of the appended claims.

CLAIMS

1. A method of detecting the presence of a transmissible spongiform encephalopathy (TSE) in an animal comprising:

(a) detecting the presence or amount of 14-3-3 isoforms in the animal's cerebrospinal fluid (CSF);

(b) comparing the presence and amount of 14-3-3 isoforms in the animal's CSF with the presence and amount of 14-3-3 isoforms in normal CSF,

wherein changes in the presence or amount or number of 14-3-3 isoforms in the animal's CSF is indicative of the TSE.

2. The method according to claim 1 wherein the 14-3-3 isoforms are detected using antibodies.

3. The method according to claim 2 wherein the antibodies are polyclonal.

4. The method according to claim 3 wherein the antibodies are monoclonal.

5. The method according to claim 2 wherein the antibodies are selected from the group consisting of antibodies against 14-3-3β, 14-3-3γ, 14-3-3δ, 14-3-3ε, 14-3-3θ, 14-3-3ζ and 14-3-3η.

6. The method according to claim 1 wherein the transmissible spongiform encephalopathy is selected from the group consisting of Creutzfeldt-Jakob disease (CJD), sporadic CJD, new variant CJD, Gerstmann-Staussler-Scheinker syndrome, Fatal Familial Insomnia, Kuru, sheep scrapie, bovine spongiform encephalopathy, transmissible mink encephalopathy, and chronic wasting disease of captive mule deer and elk.

7. The method according to claim 1 wherein the presence or amount of 14-3-3 isoforms is detected using 2-dimensional gel electrophoresis.

8. The method according to claim 2 wherein the antibody is derived from native 14-3-3.

9. The method according to claim 2 wherein the antibody is derived from a synthetic peptide.

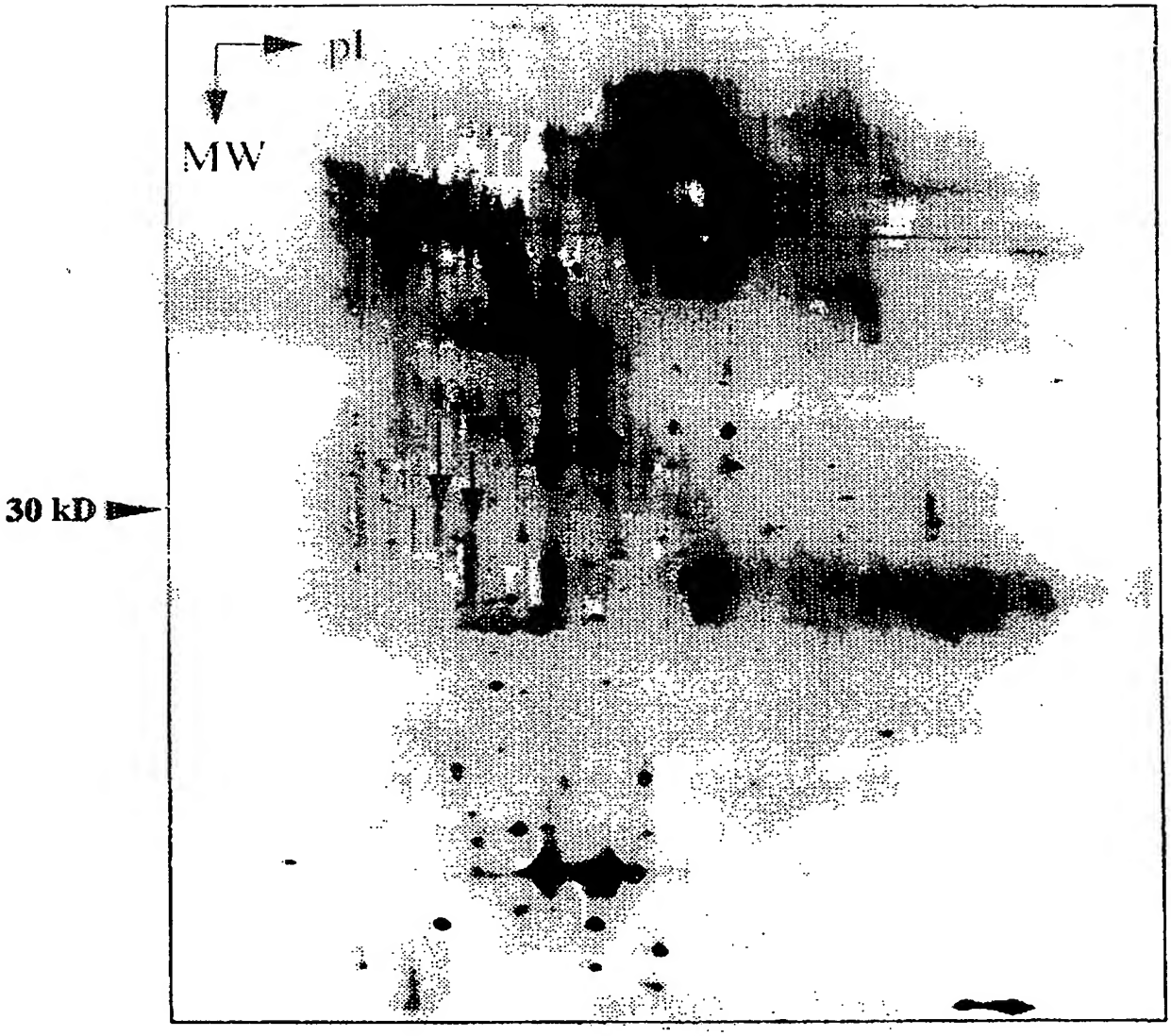
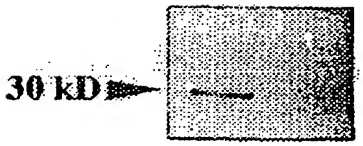


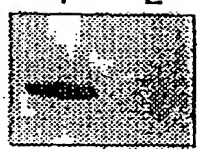
Figure 1

Fig. 2A



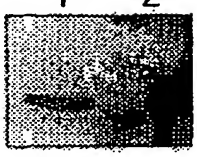
14- 3- 3

Fig. 2B



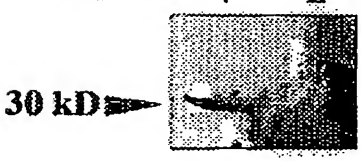
14- 3- 3γ

Fig. 2C



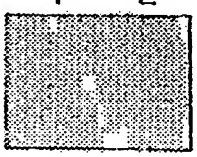
14- 3- 3β

Fig. 2D



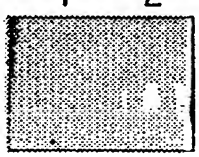
14- 3- 3θ

Fig. 2E



14- 3- 3ε

Fig. 2F



14- 3- 3ζ

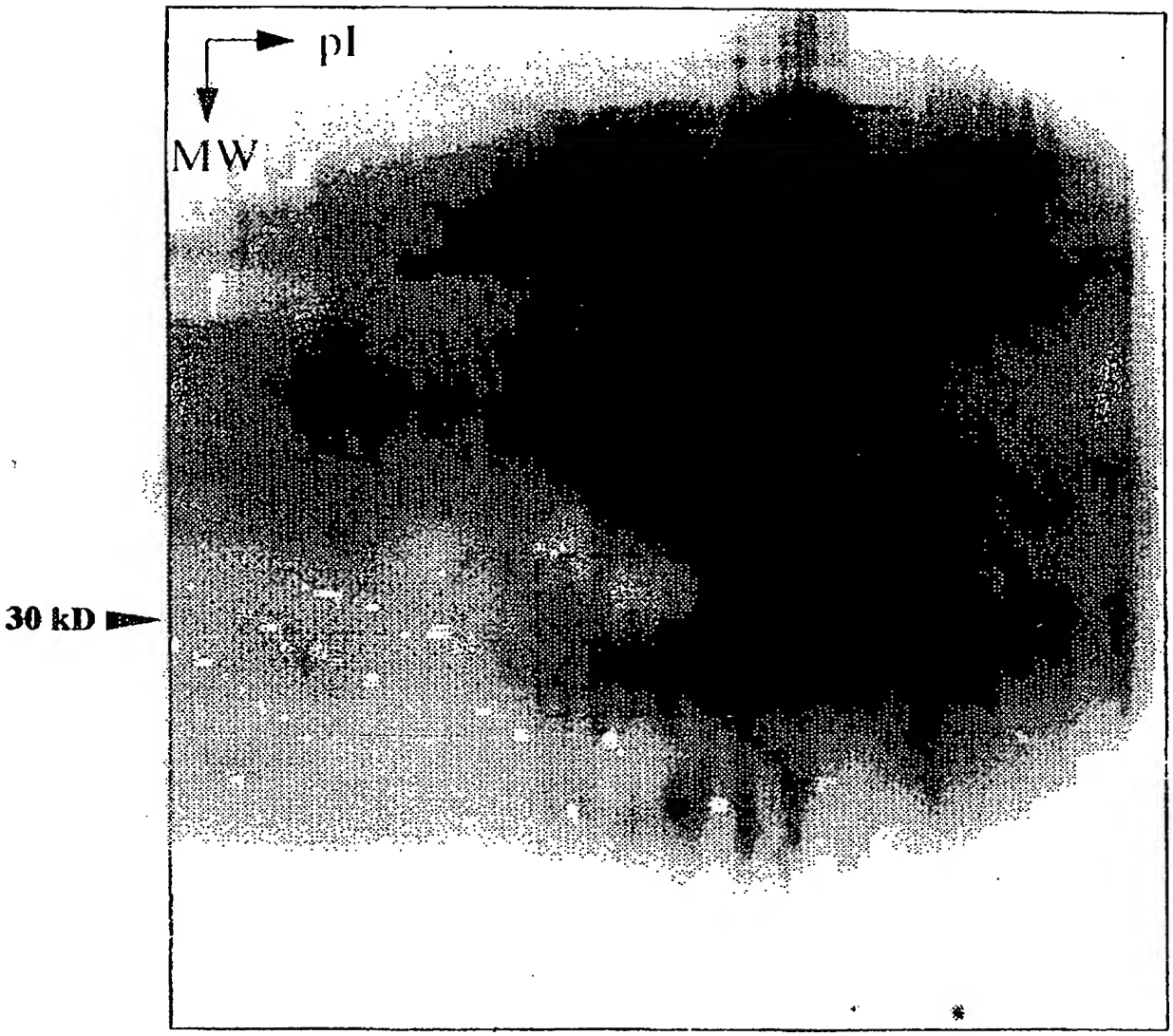


Figure 3

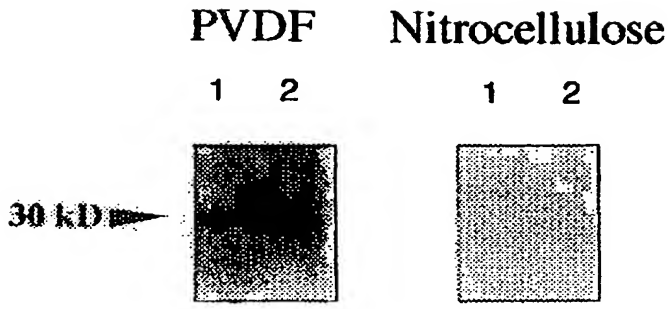


Figure 4

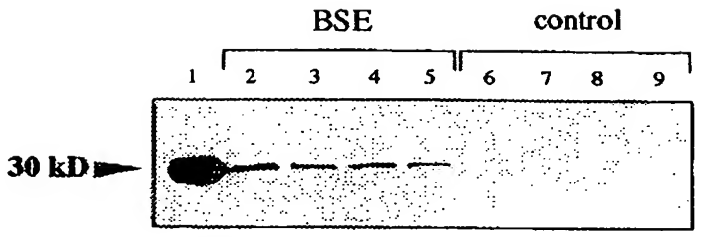


Figure 5

INTERNATIONAL SEARCH REPORT

Int. tional Application No
PCT/US 97/22820

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 G01N33/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	HSICH G. ET AL: "The 14-3-3 brain protein in cerebrospinal fluid as a marker for transmissible spongiform encephalopathies." THE NEW ENGLAND JOURNAL OF MEDICINE, vol. 335, no. 13, 26 September 1996, pages 924-930, XP002063494 see the whole document ---	1-9
Y	MARTIN H. ET AL: "Antibodies against the major brain isoforms of 14-3-3 protein.An antibody specific for the N-acetylated amino-terminus of a protein." FEBS LETTERS, vol. 331, no. 3, October 1993, pages 296-303, XP002063495 see the whole document ---	1-9
	-/--	

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

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Date of the actual completion of the international search	Date of mailing of the international search report
27 April 1998	18/05/1998

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Moreno, C

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INTERNATIONAL SEARCH REPORT

Int .tional Application No
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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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